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Spore Cortex Hydrolysis Precedes Dipicolinic Acid Release during *Clostridium difficile* Spore Germination

Michael B. Francis, Charlotte A. Allen, Joseph A. Sorg

Department of Biology, Texas A&M University, College Station, Texas, USA

ABSTRACT

Bacterial spore germination is a process whereby a dormant spore returns to active, vegetative growth, and this process has largely been studied in the model organism *Bacillus subtilis*. In *B. subtilis*, the initiation of germinant receptor-mediated spore germination is divided into two genetically separable stages. Stage I is characterized by the release of dipicolinic acid (DPA) from the spore core. Stage II is characterized by cortex degradation, and stage II is activated by the DPA released during stage I. Thus, DPA release precedes cortex hydrolysis during *B. subtilis* spore germination. Here, we investigated the timing of DPA release and cortex hydrolysis during *Clostridium difficile* spore germination and found that cortex hydrolysis precedes DPA release. Inactivation of either the bile acid germinant receptor, *cspC*, or the cortex hydrolase, *sleC*, prevented both cortex hydrolysis and DPA release. Because both cortex hydrolysis and DPA release during *C. difficile* spore germination are dependent on the presence of the germinant receptor and the cortex hydrolase, the release of DPA from the core may rely on the osmotic swelling of the core upon cortex hydrolysis. These results have implications for the hypothesized glycine receptor and suggest that the initiation of germinant receptor-mediated *C. difficile* spore germination proceeds through a novel germination pathway.

IMPORTANCE

***Clostridium difficile* infects antibiotic-treated hosts and spreads between hosts as a dormant spore. In a host, spores germinate to the vegetative form that produces the toxins necessary for disease. *C. difficile* spore germination is stimulated by certain bile acids and glycine. We recently identified the bile acid germinant receptor as the germination-specific, protease-like CspC. CspC is likely cortex localized, where it can transmit the bile acid signal to the cortex hydrolase, SleC. Due to the differences in location of CspC compared to the *Bacillus subtilis* germinant receptors, we hypothesized that there are fundamental differences in the germination processes between the model organism and *C. difficile*. We found that *C. difficile* spore germination proceeds through a novel pathway.**

Clostridium difficile (a Gram-positive, spore-forming, strict anaerobe) has become a significant threat to antibiotic-treated or immunocompromised hosts. Antibiotics are known to disrupt the colonic microbiota, and this perturbation permits *C. difficile* colonization (1, 2). Due to the strict anaerobic nature of *C. difficile* cells, spores are generally thought to be the infectious agent (only the spore can survive for extended periods of time in the aerobic environment outside a host) (3, 4). Because the spore form is noninfectious, spores must germinate to actively growing bacteria which initiate infection (5, 6). Thus, germination by *C. difficile* spores represents one of earliest steps in the pathogenesis of this organism.

Endospore germination has been extensively studied in *Bacillus* sp. and, more recently, in clostridia (7, 8). In the spore core, small acid soluble proteins help protect the chromosomal DNA, and much of the water is replaced by pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) as a 1:1 chelate with calcium (CaDPA), accounting for ca. 10% of the dry weight of the spore (8). Surrounding the spore core, is an inner spore membrane, a thin layer of cell wall peptidoglycan, a thick layer of specialized cortex peptidoglycan, an outer spore membrane, and spore coat proteins. These features help protect the spore from environmental hardship and help the spore remain in a metabolically dormant state (8). Even though spores are metabolically dormant, they interact with the environment and germinate when conditions become favorable for vegetative growth.

In *Bacillus subtilis*, germinant receptor-mediated germination can be divided into two stages. Stage I is triggered when germinant receptors embedded within the inner spore membrane respond to the presence of small molecule germinants (8). The most often described germinants for *B. subtilis* spores are L-alanine (or L-valine) or a mixture of L-asparagine, glucose, fructose, and potassium ions (AGFK) (8). The interaction of L-alanine/valine with the GerAA-AB-AC germinant receptor or AGFK with the GerB/GerK germinant receptor leads to the release of CaDPA from the core, likely through the SpoVA channel, in exchange for water (8). The release of CaDPA from the core completes stage I.

Stage II is activated by the release of CaDPA from the core during stage I, and stage II can be directly activated by an abundance of exogenous CaDPA (non-nutrient-mediated spore germination) (8). During stage II, cortex is degraded by the spore

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Address correspondence to Joseph A. Sorg, jsorg@bio.tamu.edu.

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cortex lytic enzymes (SCLEs) CwlJ and SleB (8). While the mechanism of activation of SleB is unknown, CwlJ activity is activated by DPA (8). Thus, CaDPA release from the core stimulates cortex hydrolysis, which leads to the swelling of the germ cell wall and core expansion. The expansion of the core results in further hydration of the core and complete CaDPA release (8). Upon completing stage II, spores have lost most of their resistances and are no longer considered dormant. Then, in what has been described as "ripening," the germinated spore prepares for the outgrowth of a vegetative cell (9).

C. difficile spore germination is stimulated by a combination of cholic acid derivatives and glycine (10, 11) and inhibited by chenodeoxycholic acid derivatives (10, 12–15). Although many of the ultrastructural features of the spore are conserved between *B. subtilis* and *C. difficile*, there are many differences (5, 16). Significantly, *C. difficile* does not encode the classical *ger*-type germinant receptor (17). Also, *C. difficile* encodes a single SCLE, SleC (17, 18). *C. difficile* SleC is synthesized in the mother cell during spore formation as a preproprotein and *sleC* is required for colony formation by *C. difficile* spores (18, 19). The presequence is cleaved off, presumably during transport across the spore outer membrane. The proprotein remains inactive in the dormant spore until it is cleaved by a germination-specific protease, CspB (20). In *C. difficile*, *cspB* is encoded as a fusion to *cspA* (17, 20). Upon translation of the *cspBA* mRNA, CspBA undergoes interdomain cleavage to generate both CspB and CspA proteins (20). A third protein, CspC, is encoded downstream of *cspBA* (17, 20). In *C. perfringens*, all three Csp proteins have predicted catalytic activity (all three possess intact catalytic triads) (21, 22). In *C. difficile*, only CspB is predicted to have catalytic activity because the residues important for catalysis are mutated in *cspA* and *cspC* (6, 17, 20). Recently, we identified *C. difficile* CspC as the bile acid germinant receptor (6). Certain SNPs in *C. difficile cspC* can abrogate spore germination, while other SNPs alter germinant specificity (6). We proposed a model where CspC activates CspB proteolytic activity and CspB cleaves pro-SleC to an active form. Activated SleC then begins to degrade the *C. difficile* spore cortex (6). Because the *C. difficile* germinant receptor complex (CspA, CspB, CspC, and SleC) is likely located in or near the spore cortex, while the *B. subtilis* germinant receptor complex is located in the spore's inner membrane, we hypothesized that there may be fundamental differences between the mechanisms of germinant receptor-mediated *C. difficile* spore germination and *B. subtilis* spore germination.

We investigated how *C. difficile* spores germinate with respect to the proposed stages of germination, as described for *B. subtilis*. In contrast to what is observed for *B. subtilis* spore germination (and *C. perfringens* [23]), we found that cortex hydrolysis preceded DPA release during *C. difficile* spore germination. Significantly, mutations in either the *C. difficile* bile acid germinant receptor, CspC, or the cortex hydrolase, SleC, prevented both cortex hydrolysis and DPA release by germinating *C. difficile* spores. These results suggest that DPA release during *C. difficile* spore germination may be entirely dependent on core swelling or changes to cortex peptidoglycan and that the hypothesized glycine germinant receptor is likely not located in the spore inner membrane.

MATERIALS AND METHODS

Bacteria and strains. Wild-type *C. difficile* UK1 (6, 12, 15) and *C. difficile* M68 (15, 24, 25), *C. difficile* JSC10 (*cspC*::*ermB*) (6), and *C. difficile* CAA5 (*sleC*::*ermB*) were routinely grown in an anaerobic atmosphere (10% H_2, 5% CO_2, 85% N_2) at 37°C in brain heart infusion agar supplemented with 5 g/liter yeast extract and 0.1% L-cysteine (BHIS). *B. subtilis* PS533 and *B. subtilis* FB113 (*cwlJ*::*tet sleB*::*spc*) (26) were a generous gift from Peter Setlow and were routinely grown on Difco sporulation medium (DSM). *E. coli* DH5α was grown on Luria-Bertani (LB) medium. Chloramphenicol (20 μg/ml), thiamphenicol (10 μg/ml), lincomycin (10 μg/ml), kanamycin (50 μg/ml for *C. difficile*, 20 μg/ml for *E. coli*, and 7 μg/ml for *B. subtilis*), spectinomycin (100 μg/ml), or tetracycline (5 μg/ml for *C. difficile* and 20 μg/ml for *B. subtilis*) were added where indicated.

Molecular biology. To generate the TargeTron insertion into *C. difficile* UK1 *sleC*, we took advantage of a previously described primer set (18). The intron retargeting fragment was generated using the following primers: sleC(128a) IBS (AAAAAAGCTTATAATTATCCTTACATTACTTCT TAGTGCGCCCAGATAGGGTG), sleC(128a)EBS1d (CAGATTGTACA AATGTGGTGATAACAGATAAGTCTTCTTAGGTAACTTACCTTTCT TTGT), sleC(128a)EBS2 (TGAACGCAAGTTTCTAATTTCGGTTTAAT GTCGATAGAGGAAAGTGTCT), and EBS universal (CGAAATTAGAA ACTTGCGTTCAGTAAAC) using SOE-PCR as describe in the TargeTron manual (Sigma-Aldrich, St. Louis, MO). The 350-bp fragment was cloned into pCR2.1-TOPO (Life Technologies, Carlsbad, CA) to yield pCA2, and the sequence of the insert was verified. The 350-bp fragment was subcloned into the HindIII/BsrGI sites of the pJS107 TargeTron shuttle vector (15) to yield pJS113. The pJS113 plasmid was introduced into *B. subtilis* Bs49 using standard techniques. The pJS113 shuttle vector was introduced into *C. difficile* UK1 via conjugal transfer from *B. subtilis* Bs49, as described previously (6). Tetracycline-sensitive, thiamphenicol-resistant (Tn*916* transposon-negative, plasmid-positive) strains were identified. These isolates were then spread on BHIS medium supplemented with lincomycin to select for the TargeTron insertion into *sleC*. Lincomycin-resistant colonies were screened by PCR for the presence of the TargeTron insertion into *sleC*, as described previously (18). Isolates with the insertion were frozen down as *C. difficile* CAA5 and have the expected phenotype of an *sleC* mutant (inability of spores to form colonies on BHIS agar supplemented with taurocholic acid) (18).

C. difficile CAA5 was complemented by expressing *sleC* in *trans* from the pJS116 shuttle vector (a pMTL84151 derivative) (6, 27). The *C. difficile* UK1 *sleC* gene and promoter region were amplified using the primers 5′sleC_Gibson (TACGAATTCGAGCTCGGTACCCGGGGATCCGATT ATTTTCCTTTCAAAATTTTTGATTTATTTATGATTTATATC) and 3′sleC_Gibson (AGTGCCAAGCTTGCATGTCTGCAGGCCTCGAGTT AAATTAAAGGATTTAAAGAAGCTATTCTAGTTGTAG) and Phusion DNA polymerase (New England BioLabs, Beverly, MA). The resulting fragment was introduced into pJS116 between the BamHI and XhoI restrictions sites using Gibson assembly (28). The resulting plasmid, pMF02, was introduced into *C. difficile* CAA5 as described above.

Spore formation. *C. difficile* spores were generated as described previously (6, 12, 15, 29). *B. subtilis* vegetative cells were spread on DSM agar medium for spore production (30). After 2 days, growth was harvested by scraping the plates and suspending the samples in water. This suspension (containing vegetative cells, cell debris, and spores) was then heated to 75°C for 1 h to melt any agar that was scraped with spores. The suspension was centrifuged for 10 min at room temperature and 3,000 × *g*. The supernatant was removed, and the pellet was resuspended in 10 ml of sterile water. To purify the spores from the vegetative cells and cell debris, the resuspended samples were layered onto a gradient of 10 ml of 20% HistoDenz (wt/vol), and 10 ml of 50% HistoDenz (wt/vol) and centrifuged for 1 h at 4°C and 18,900 × *g*. The supernatant was then removed, and the spore pellet was resuspended in 1 ml of water. The purified spores were then washed five times in water by centrifuging for 1 min at room temperature and 14,000 × *g*.

Monitoring the initiation of spore germination. The initiation of spore germination was monitored aerobically at 600 nm (the initiation of *C. difficile* spore germination is unaffected by the presence of oxygen). To initiate *B. subtilis* spore germination, purified spores were suspended in 10

mM Tris (pH 8.4) and 100 mM L-valine. *C. difficile* spore germination was initiated by suspending spores in 10 mM Tris (pH 7.5), 150 mM NaCl, 100 mM glycine, and 10 mM taurocholic acid. Spores were heat shocked at either 80°C for *B. subtilis* or 65°C for *C. difficile* for 30 min and then placed on ice. Then, 5 μl of spores was diluted into 995 μl of buffer with or without germinant and mixed, and the change in optical density at 600 nm (OD_{600}) was measured.

Monitoring CaDPA release. CaDPA release was monitored in real time using terbium fluorescence (31). An opaque, 96-well plate was prepared with the 125 μl of the germination solutions (see above) supplemented with 800 μM $TbCl_3$. Heat-activated spores were then sedimented for 1 min at 14,000 × *g* and resuspended in an equal volume of water to remove any CaDPA that may have released due to autogerminating spores. A 5-μl sample of a spore suspension (OD_{600} of 60) was added to each well, and the CaDPA release was monitored by using a Molecular Devices Spectramax M3 fluorescence plate reader (Molecular Devices, Sunnyvale, CA) (excitation, 270 nm; emission, 545 nm; cutoff, 420 nm [appropriate wavelengths for the DPA-Tb^{3+} complex]). For experiments involving mutations in the germination pathway (i.e., *B. subtilis cwlJ::tet sleB::spc* or *sleB*, *C. difficile cspC::ermB*, or *C. difficile sleC::ermB*), the amount of CaDPA released was compared to that of the wild-type strain.

Assaying cortex fragment release by germinating spores. Cortex fragments were detected using an assay based on the presence of reducing sugars in the germination medium, as described previously (32, 33). Briefly, *B. subtilis* spores or *C. difficile* spores were heat activated, as described above, and stored on ice until use. An 11-ml germination solution (see above) was prepared. Before beginning the assay, a 1.0-ml sample was drawn to serve as a blank for cortex fragment detection, and a separate 100-μl sample was taken as a blank for measuring DPA release. A target spore density (OD_{600}) of ~3.0 yielded the best results for detecting cortex fragments. A zero time point sample was taken immediately after the addition of spores and centrifuged for 1 min at 14,000 × *g*. Then, 1.0 ml of this sample was transferred to a fresh tube for cortex fragment analysis (see below), and 100 μl was taken to monitor the amount of CaDPA released. This procedure was repeated at selected time points until the experiment was completed. After all time point samples were collected, the samples were frozen at −80°C and lyophilized.

Lyophilized samples were resuspended in 120 μl of 3N HCl supplemented with 1% phenol and 0.5% β-mercaptoethanol and then transferred to 2-ml screw-cap tubes. Samples were then placed in a 95°C recirculating water bath for 4 h. After incubation, the samples were placed on ice until cool and neutralized with 120 μl of 3 M NaOH. To these samples 80 μl of a saturated sodium bicarbonate solution and 80 μl of a 5% acetic anhydride solution were added, and the samples were mixed. The samples were incubated at room temperature for 10 min and then transferred back to the 95°C water bath for 3 min. The samples were removed from the water bath and cooled on ice, and then 400 μl of 6.54% $K_2B_4O_7{\cdot}4H_2O$ was added to each tube, followed by mixing. The resulting solution was then heated for 7 min in the 95°C water bath and placed on ice for 5 min, during which the color reagent was made. This color reagent was made by dissolving 0.320 g of *p*-dimethylaminobenzaldehyde in 1.9 ml of glacial acetic acid. After the *p*-dimethylaminobenzaldehyde was completely dissolved, 100 μl of 10 N HCl was added, the solution was mixed, and then 5 ml of glacial acetic acid was added. Next, 100 μl of each cooled cortex sample was transferred to a new 1.5-ml microcentrifuge tube, and 700 μl of the color solution was added. Samples were incubated in a 37°C water bath for 20 min. After incubation, 200 μl of each sample was transferred to a clear 96-well plate and quantified at 585 nm using a Molecular Devices Spectramax M3 fluorescence plate reader. As a positive control for reducing sugar detection, a standard curve was generated in each experiment using 0, 12.5, 25, 50, 100, 250, 500, and 5,000 nmol of *N*-acetylglucosamine. For experiments involving mutations in the germination pathway (i.e., *B. subtilis cwlJ::tet sleB::spc*, *C. difficile cspC::ermB*, or *C. difficile sleC::ermB*), the amount of cortex released was compared to that of the wild-type strain.

Statistical analysis. Data points represent the mean from three independent experiments, and error bars represent the standard deviations from the mean. Statistical analysis between time points, where indicated, was performed using a two-tailed Student *t* test.

RESULTS

Comparison of the initiation of *C. difficile* and *B. subtilis* spore germination. *B. subtilis* spore germination can be triggered via several pathways (e.g., nutrient-mediated activation of the *ger*-type germinant receptors or direct activation of cortex hydrolysis by CaDPA). To begin to understand the events that occur during *C. difficile* spore germination, we compared *C. difficile* spore germination to that of *B. subtilis* spore germination via activation of their respective germinant receptors. During spore germination, spores transition from a phase-bright state (dormant) to a phase-dark state (loss of dormancy). This transition can be monitored spectrophotometrically by measuring the OD_{600} of pure spore suspensions incubated under different conditions. When spores respond to germinant, they release their large depot of CaDPA from the core. This action results in a large and rapid decrease in the OD_{600} of the spore suspension (34). *C. difficile* UK1 spores were suspended in buffer supplemented with either taurocholic acid and glycine or taurocholic acid alone, and germination was monitored at 600 nm. As described previously, spores rapidly germinated upon exposure to both taurocholic acid and glycine but not when exposed to taurocholic acid alone (Fig. 1A and data not shown) (10). Although the use of absorbance to monitor germination is convenient, it is not a quantitative measure of CaDPA release and can include cortex hydrolysis at later time points (34). Thus, to provide a quantitative measure of DPA release, we monitored CaDPA release in real-time using an assay based on terbium fluorescence (31, 35). *C. difficile* spores released CaDPA in the presence of taurocholic acid and glycine but not in response to taurocholic acid alone (Fig. 1A and data not shown) and completed DPA release in ~30 min (no further increase in DPA occurred after 30 min).

To compare *C. difficile* spore germination to that of *B. subtilis*, we incubated purified *B. subtilis* spores in buffer supplemented with L-valine. As described previously, the absorbance of the spore suspension decreased when incubated in the presence of L-valine and not in the absence of L-valine (Fig. 1B and data not shown) (36). Similarly, Tb^{3+} fluorescence increased when *B. subtilis* spores were suspended in buffered L-valine (indicating CaDPA release) but not in buffer alone (Fig. 1B and data not shown).

So that we could directly compare the absorbance assay to the terbium fluorescence assay, we plotted the percent change from the absorbance assay and the percent maximum Tb^{3+} fluorescence on the same graph. When analyzed in this manner, changes in the absorbance of germinating *C. difficile* spores occurred much earlier than changes observed in Tb^{3+} fluorescence (CaDPA release) (Fig. 1C). During *B. subtilis* spore germination, the CaDPA release curve closely followed that of the absorbance curve, confirming a previous study which demonstrated that much of the absorbance change is due to the released CaDPA (Fig. 1D) (34). Because CaDPA release is one of the first measurable events during germinant receptor-activation of *B. subtilis* spore germination, these results suggest that there may be events occurring during *C. difficile* spore germination before CaDPA is released.

CaDPA release precedes cortex hydrolysis release during *B. subtilis* spore germination. When CaDPA is released from the

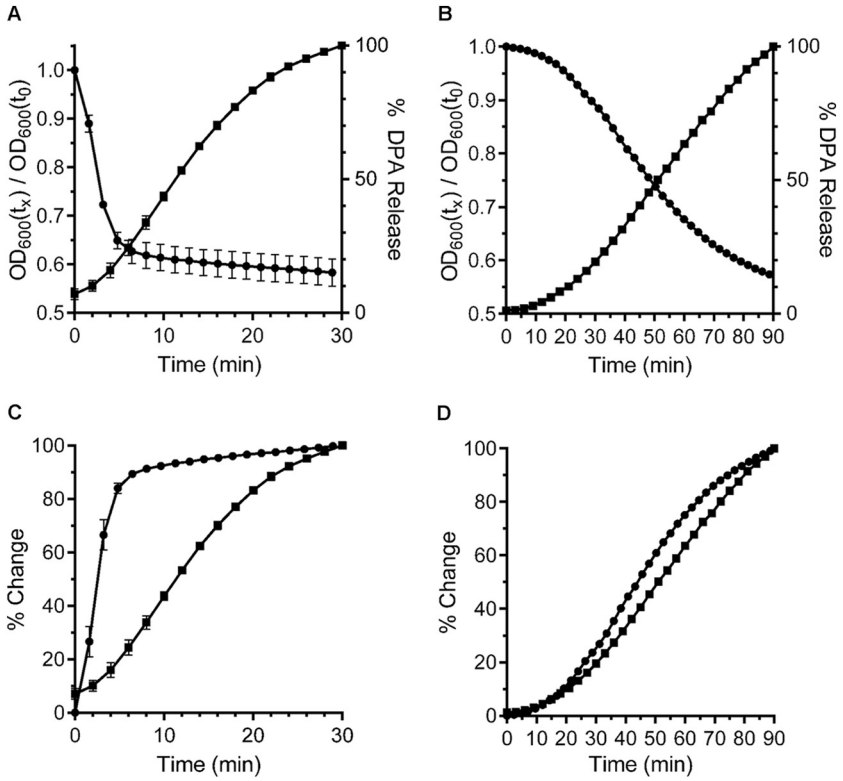

FIG 1 Comparison of the initiation of *C. difficile* and *B. subtilis* spore germination. (A) Purified *C. difficile* UK1 spores were suspended in buffer supplemented with taurocholic acid and glycine. Germination was monitored by plotting the ratio of the OD_{600} at a given time to the OD_{600} at time zero (●) and DPA release from germinating *C. difficile* spores was monitored using Tb^{3+} fluorescence and normalized to the maximum amount of DPA released in the indicated time frame (■). (B) Purified *B. subtilis* PS533 spores were suspended in buffer supplemented with L-valine and germination was monitored as described above. The data from the OD_{600} in panels A and B were converted to the percent change so that the curves could be directly compared. The converted OD_{600} data were plotted with the DPA release data in panels C and D, respectively. The data represent the averages from three independent experiments, and error bars represent the standard deviations.

spore core, it transits through the spore cortex and activates the spore cortex hydrolase, CwlJ. CwlJ activity (and SleB activity) leads to the release of cortex fragments into the surrounding germination medium. *B. subtilis* spores suspended in germination buffer supplemented with L-valine released most of their CaDPA within 2.5 h (Fig. 2A). Spores suspended in buffer alone did not release CaDPA (data not shown). When we monitored for the presence of cortex fragments (as measured by the abundance of reducing sugars in the germination solution), we observed that these cortex fragments appeared after CaDPA is released (Fig. 2A) and that their presence was dependent on L-valine (data not shown). These results confirm the previous observations that CaDPA release precedes cortex hydrolysis in *B. subtilis* and, importantly, that we can detect cortex fragments during spore germination (34).

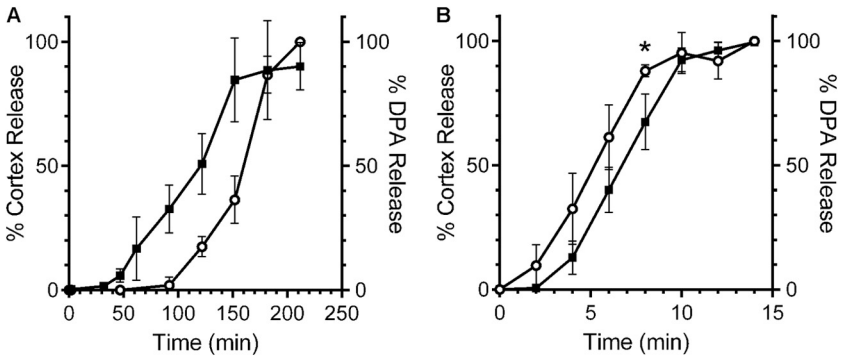

FIG 2 Comparing the release of cortex fragments and CaDPA from germinating *B. subtilis* and *C. difficile* spores. (A) Purified *B. subtilis* PS533 spores were suspended in buffer with L-valine. (B) Purified *C. difficile* UK1 spores were suspended in buffer with taurocholic acid and glycine. At the indicated time points, a sample was taken, and the amounts of cortex fragments (○) and CaDPA (■) in the germination solutions were determined. The data represent the averages from three independent experiments, and error bars represent the standard deviations. *, $P < 0.04$.

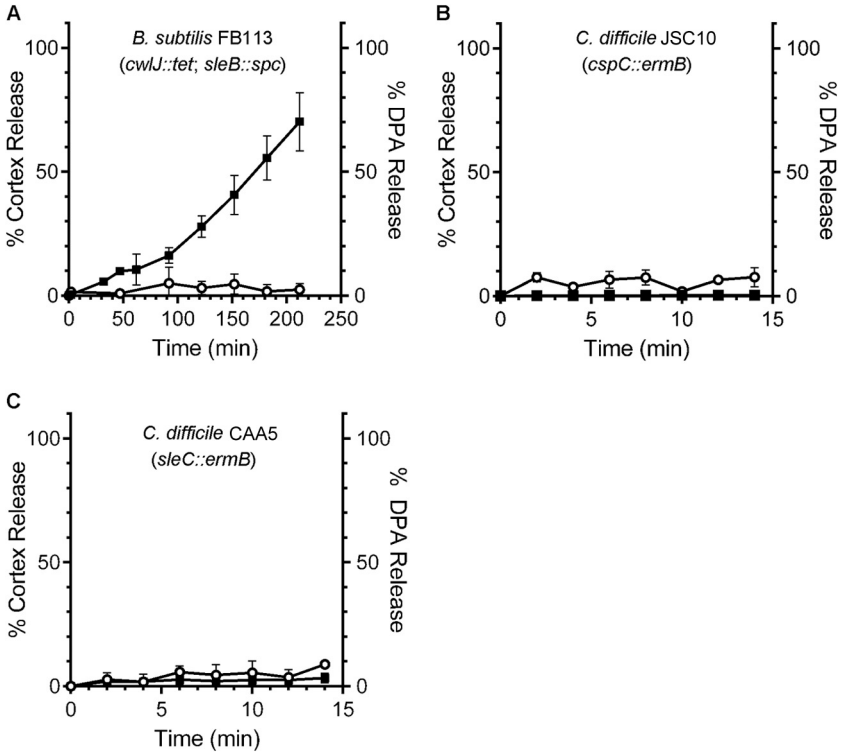

FIG 3 Genetic analysis of cortex hydrolysis and CaDPA release from germinating *B. subtilis* and *C. difficile* spores. (A) Purified *B. subtilis* FB113 (*cwlJ*::*tet sleB*::*spc*) spores were suspended in buffer supplemented with L-valine. (B and C) *C. difficile* JSC10 (*cspC*::*ermB*) spores (B) and *C. difficile* CAA5 (*sleC*::*ermB*) spores (C) were suspended in buffer supplemented with taurocholic acid and glycine. At the indicated time points, a sample was taken, and the amounts of cortex fragments (○) and CaDPA (■) in the germination solution were determined. The data represent the averages from three independent experiments, and error bars represent the standard deviations.

Cortex hydrolysis precedes CaDPA release during *C. difficile* spore germination. Based on our observations that the optical density of germinating *C. difficile* spores decreased before the appearance of CaDPA in solution (Fig. 1C) and that the newly identified bile acid germinant receptor is likely localized in the cortex (6), we hypothesized that cortex hydrolysis may precede CaDPA release during *C. difficile* spore germination. *C. difficile* UK1 spores were suspended in germination buffer supplemented with taurocholic acid only or both taurocholic acid and glycine and assayed for the presence of both cortex fragments and DPA in the germination solution. Interestingly, we detected cortex fragment release from the germinating spores within 2 min of germination (the earliest time point we can measure). At this time point, CaDPA is either not released or below the limit of detection (Fig. 2B). At 4 min, the rate of cortex fragment release increased. During this time CaDPA begins to be released and followed closely the curve for the cortex fragments, which remained steady until 8 min after the initiation of germination (Fig. 2B). At 8 min, there was a statistically significant difference between the cortex fragment curve and the CaDPA curve ($P < 0.04$). Taken together, these results suggest that *C. difficile* spore cortex hydrolysis precedes CaDPA release during germination.

To control for the observed differences in cortex hydrolysis and CaDPA release, we analyzed cortex hydrolysis in *B. subtilis* FB113, a strain with engineered mutations in both cortex hydrolases (*sleB* and *cwlJ*). When *B. subtilis* FB113 spores were suspended in buffer supplemented with L-valine, spores released CaDPA (Fig. 3A). However, inactivating both *sleB* and *cwlJ* cortex hydrolases resulted in the inability of these *B. subtilis* spores to hydrolyze cortex in response to L-valine (Fig. 3A).

Conversely, when *C. difficile cspC* spores are suspended in buffer supplemented with taurocholic acid and glycine, neither cortex fragments nor CaDPA are released (Fig. 3B); CaDPA release was restored by expressing *cspBAC* in *trans* (6). Further, inactivating the lone *C. difficile* SCLE, *sleC*, also prevented cortex hydrolysis and CaDPA release (Fig. 3C); germination (measured both by OD_{600} level and CaDPA release) was restored by expressing in *trans* a copy of *C. difficile sleC* (see Fig. S1 in the supplemental material). Because *C. difficile cspC* still expresses the SleC cortex hydrolase, but CaDPA and cortex fragments are not released, these results suggest that cortex hydrolysis and CaDPA release during *C. difficile* spore germination are coupled.

Analyzing spore germination in another *C. difficile* strain. It was previously reported that there may be heterogeneity among *C. difficile* isolates in terms of their germination responses (37, 38). Therefore, we analyzed how cortex hydrolysis and CaDPA release occurs in another *C. difficile* ribotype. As described above for *C. difficile* UK1, when *C. difficile* M68 spores are suspended in buffer containing taurocholic acid and glycine (but not taurocholic acid only [data not shown]), cortex fragments appeared in the germination solution before CaDPA is detected (Fig. 4). During *C. difficile* M68 spore germination, at the earliest time point of 2 min, there was a difference between cortex fragments and CaDPA ($P < 0.001$) (Fig. 4). These results support the idea that cortex hydro-

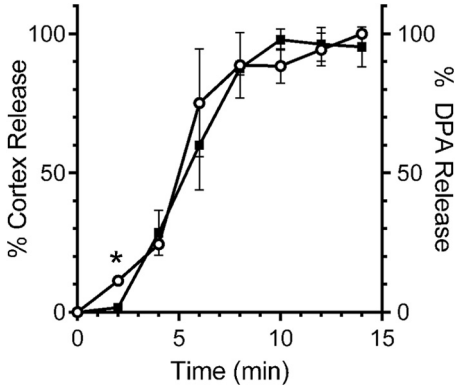

FIG 4 Cortex hydrolysis precedes CaDPA release during *C. difficile* M68 spore germination. Purified *C. difficile* M68 spores were suspended in buffer with taurocholic acid and glycine. At the indicated time points, a sample was taken, and the amounts of cortex fragments (○) and CaDPA (■) in the germination solution were determined. The data represent the averages from three independent experiments, and error bars represent the standard deviations. *, $P < 0.001$.

lysis preceding CaDPA release is a general phenomenon during *C. difficile* spore germination and not specific to one isolate. Our results clearly show that the initiation of germinant receptor-mediated *C. difficile* spore germination occurs through a novel pathway.

DISCUSSION

Germination by *C. difficile* spores seems to occur differently than for other spore-forming bacteria. Upon sequencing and analysis of the *C. difficile* genome, it was apparent that *C. difficile* did not encode orthologues of the *ger*-type germinant receptors found in other spore-forming bacteria. This suggested that *C. difficile* spores may germinate in response to unique germinants or use novel mechanisms to initiate spore germination or both (17).

It has been known for approximately 30 years that certain bile acids stimulate *C. difficile* spore germination (39, 40). Although much work has focused on the signals that can stimulate or inhibit *C. difficile* spore germination, the proteins that responded to these signals had remained elusive. In a genetic screen to select for *C. difficile* mutants whose spores do not respond to taurocholic acid as a germinant, we identified CspC as the bile acid germinant receptor (6). Due to the differences between the predicted locations of the *C. difficile* germinant receptor complex (CspC, CspB, and SleC) and the locations of the *B. subtilis* spore germinants receptors (GerAA-AB-AC), we hypothesized that *C. difficile* spore germination may occur differently than that observed in the model organism.

We observed that *C. difficile* spore germination is not initiated in the same manner, as observed for *B. subtilis*. We found that cortex hydrolysis precedes CaDPA release during germination by *C. difficile* spores and that this seems to be a general phenomenon among *C. difficile* isolates; the *C. difficile* M68 strain, a different ribotype, also released cortex fragments before CaDPA. Unlike what is observed for *B. subtilis* spore germination, we could not genetically separate cortex hydrolysis from CaDPA release by inactivating either the bile acid germinant receptor or the SCLE. Both *C. difficile cspC* and *C. difficile sleC* are required to hydrolyze cortex and, without cortex hydrolysis, the release of CaDPA from the core in is not observed (Fig. 3B and C, respectively).

Interestingly, *C. perfringens* encodes orthologues of both the classical *ger*-type germinant receptor, the Csp proteases and SleC. However, *C. perfringens* does not germinate in response to bile acids (41, 42). Although there seems to be conservation in the Csp proteases, *C. perfringens* CspA, CspB, and CspC are catalytically active proteases that could activate SleC to stimulate cortex hydrolysis (21, 22, 32). Mutations in *C. perfringens sleC* result in strains that still release CaDPA but do not hydrolyze cortex (23). Thus, our observation that cortex hydrolysis precedes CaDPA release during *C. difficile* spore germination is not a general phenomenon among all clostridia. However, this may be a novel mechanism for stimulating germination in spore-forming bacteria that do not encode the classical *ger*-type germinant receptor.

If mutations in the bile acid germinant receptor prevent both cortex hydrolysis and CaDPA release from the core, how is CaDPA release mediated during *C. difficile* spore germination? In our working model (Fig. 5), taurocholic acid interacts with CspC, which transmits the bile acid signal to CspB. Activated CspB, in turn, cleaves pro-SleC to an active hydrolase, which begins to hydrolyze cortex, releasing cortex fragments into the surrounding milieu. Cortex hydrolysis allows the germ cell wall to expand and, with it, the inner spore membrane. In our model, either an un-

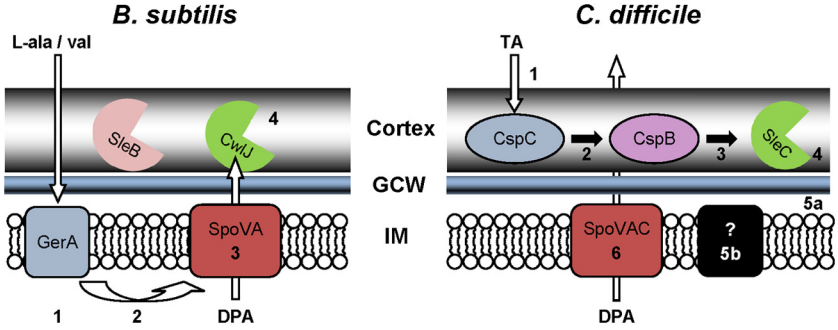

FIG 5 Models for spore germination. (A) During the initiation of *B. subtilis* spore germination, L-alanine (or L-valine) interacts with the GerA germinant receptor complex (location 1). The SpoVA channel (which includes the SpoVAD DPA-binding protein) is then activated (location 2), and it releases CaDPA from the spore core (3). Released CaDPA activates the CwlJ cortex hydrolase (location 4) triggering cortex hydrolysis. (B) The initiation of *C. difficile* spore germination is triggered when the bile acid germinant receptor, CspC, interacts with taurocholic acid (location 1). Activated CspC then activates the germination-specific protease, CspB (2), which processes pro-SleC to an active form (location 3), and cortex hydrolysis begins (location 4). Then, due to either core swelling (location 5a) or through the action of an unknown protein (location 5b), SpoVAC releases CaDPA (location 6).

identified protein responds to the cell wall expansion and triggers CaDPA release, or the expansion of the inner spore membrane alone triggers CaDPA release. This suggests that a mechanosensitive channel is responsible for the release of CaDPA during *C. difficile* spore germination. *C. difficile* encodes orthologues of several mechanosensitive proteins (e.g., *mscL* and *mscS*), and most of these are likely to be involved in maintaining osmotic homeostasis during vegetative growth and probably have no role in spore germination (17, 43–45).

In *B. subtilis*, germination is triggered through the interaction of germinants with the germinant receptors imbedded in the inner spore membrane (Fig. 5). The interaction of these germinants with their cognate receptors triggers CaDPA release, likely through the SpoVA channel (Fig. 5) (46). Then, as described above, CaDPA activates cortex hydrolysis (Fig. 5). The *B. subtilis* SpoVA complex is composed of seven different proteins: SpoVAA, SpoVAB, SpoVAC, SpoVAD, SpoVAEa, SpoVAEb, and SpoVAF. Most of these proteins are important for both CaDPA import into the developing spore during sporulation and CaDPA release during germination (e.g., SpoVAD binds DPA [47]). *C. difficile* does not encode orthologues of many of these proteins. However, *C. difficile* does encode *spoVAC*, *spoVAD* and *spoVAE*. Recently, Velasquez et al. reported a function for SpoVAC (48). These authors determined that SpoVAC is a mechanosensitive channel (48). How this protein functions during *B. subtilis* spore germination is unclear. For *C. difficile* spore germination, we propose that SpoVAC responds to the change in osmolarity that occurs upon cortex hydrolysis, and then either it alone provides a channel for CaDPA release or it is part of a larger channel that is mechanically gated.

Although the *C. difficile* bile acid germinant receptor is known, the receptor with which glycine interacts is not. We speculated that the unidentified glycine germinant receptor may be localized to the inner membrane of the spore core, thus performing a function similar to that of the *B. subtilis* germinant receptors (6). However, because a mutation in the bile acid germinant receptor prevented both cortex hydrolysis and CaDPA release, we propose that the hypothesized glycine receptor is either (i) part of the known *C. difficile* germinant receptor complex (CspB, CspA, CspC, and SleC) or (ii) located in the inner spore membrane but whose activity is dependent on CspC activity.

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